The Journal of Experimental Biology 206, 4539-4551

doi:10.1242/jeb.00706

Influences of thermal acclimation and acute temperature change on the motility of epithelial wound-healing cells (keratocytes) of tropical, temperate and Antarctic fish

Rachael A. Ream[1], Julie A. Theriot[1] and George N. Somero[2,*]

[1]*Biochemistry Department, Beckman Center, Room 473A, Stanford University School of Medicine, Stanford, CA 94305-5307, USA* and [2]*Hopkins Marine Station, Pacific Grove, CA 93950, USA*

*Author for correspondence (e-mail: somero@stanford.edu)

Accepted 4 September 2003

Summary

The ability to heal superficial wounds is an important element in an organism's repertoire of adaptive responses to environmental stress. In fish, motile cells termed keratocytes are thought to play important roles in the wound-healing process. Keratocyte motility, like other physiological rate processes, is likely to be dependent on temperature and to show adaptive variation among differently thermally adapted species. We have quantified the effects of acute temperature change and thermal acclimation on actin-based keratocyte movement in primary cultures of keratocytes from four species of teleost fish adapted to widely different thermal conditions: two eurythermal species, the longjaw mudsucker *Gillichthys mirabilis* (environmental temperature range of approximately 10–37°C) and a desert pupfish, *Cyprinodon salinus* (10–40°C), and two species from stable thermal environments, an Antarctic notothenioid, *Trematomus bernacchii* (–1.86°C), and a tropical clownfish, *Amphiprion percula* (26–30°C). For all species, keratocyte speed increased with increasing temperature. *G. mirabilis* and *C. salinus* keratocytes reached maximal speeds at 25°C and 35°C, respectively, temperatures within the species' normal thermal ranges. Keratocytes of the stenothermal species continued to increase in speed as temperature increased above the species' normal temperature ranges. The thermal limits of keratocyte motility appear to exceed those of whole-organism thermal tolerance, notably in the case of *T. bernacchii*. Keratocytes of *T. bernacchii* survived supercooling to –6°C and retained motility at temperatures as high as 20°C. Mean keratocyte speed was conserved at physiological temperatures for the three temperate and tropical species, which suggests that a certain rate of motility is advantageous for wound healing. However, there was no temperature compensation in speed of movement for keratocytes of the Antarctic fish, which have extremely slow rates of movement at physiological temperatures. Keratocytes from all species moved in a persistent, unidirectional manner at low temperatures but at higher temperatures began to take more circular or less-persistent paths. Thermal acclimation affected the persistence and turning magnitude of keratocytes, with warmer acclimations generally yielding more persistent cells that followed straighter paths. However, acclimation did not alter the effect of experimental temperature on cellular speed. These findings suggest that more than one temperature-sensitive mechanism may govern cell motility: the rate-limiting process(es) responsible for speed is distinct from the mechanism(s) underlying directionality and persistence. Keratocytes represent a useful study system for evaluating the effects of temperature at the cellular level and for studying adaptive variation in actin-based cellular movement and capacity for wound healing.

Key words: Antarctic fish, cell motility, cytoskeleton, notothenioid, keratocytes, temperature.

Introduction

Despite extensive research into thermal relationships of ectothermic animals, there remain large gaps in our understanding of how temperature affects the ability of ectotherms to cope with pathogens through both specific and nonspecific immune responses. Temperature affects both specific and nonspecific immunity, so these thermal effects are apt to be of considerable importance to ectotherms that encounter variations in environmental temperature (see Le Morvan et al., 1998 for a review). Low temperatures, consistent with normal seasonal fluctuations, correlate with increased pathogenesis in teleost fish (Baudouy et al., 1980; Bly and Clem, 1992; Le Morvan et al., 1998). Most infectious diseases occur over wide ranges of body temperatures and the outcome of the pathogenesis is likely to depend on the differential effects of change in temperature on the efficacy of the immune response and the growth rate of the pathogen. In

the case of primary antibody production, both delayed response (Rijkers et al., 1980) and suppression (Le Morvan et al., 1998) by cold temperatures have been reported. This temperature sensitivity results from the inability of T helper cells to undergo rapidly homeoviscous adaptation, leading to changes in membrane physical state that affect membrane-bound receptors and cell surface carbohydrates (Le Morvan et al., 1996). In addition to temperature mediation of the humoral response, cold temperatures also suppress lymphocyte proliferation (Ellsaesser and Clem, 1986; Bly and Clem, 1991) and specific toxicity (Verlhac et al., 1990). The effect of temperature on specific immunity is also species specific (Bly and Clem, 1992).

Less is known about the effects of temperature on nonspecific immunity, although available data indicate these effects to be complex and of potentially great importance to organisms. Low temperatures appear to increase the efficacy of certain nonspecific immune responses, including cytotoxic cell lytic activity (Le Morvan et al., 1995), phagocyte respiratory burst (Dexiang and Ainsworth, 1991; Collazos et al., 1994) and macrophage response to activating factor and burst activity (Le Morvan et al., 1997). Thus, there is evidence that nonspecific immunity may respond to cold stress relatively quickly and protect the organism from further damage by a pathogen until cold-temperature suppression of specific immunity is overcome.

A potentially important contributor to the nonspecific immune responses is the wound healing function of cells termed keratocytes, which are terminally differentiated epithelial cells found in teleosts and amphibians. The motility of these cells was first described in goldfish (*Carassius auratus*) by Goodrich (1924). Subsequent research, including *in vivo* studies using *Xenopus laevis*, showed that keratocytes migrate across wounded areas to provide a barrier to infection (Radice, 1980a,b; Euteneuer and Schliwa, 1984). Keratocytes may crawl together as a sheet of cells or move individually. The cells extend large lamellipodia, and motility is driven by actin polymerization (Theriot and Mitchison, 1991; Small et al., 1995). As is true for other types of actin-based cell motility, locomotion is thought to occur through the coordination of several distinct steps. First, the cell extends its leading edge forward, and this protrusion of the membrane is coupled with adhesion to the substrate. Next, traction force leads to a forward translocation of the cell body. Lastly, contact with the substrate at the rear of the cell is dissociated and the rear edge of the cell is released to follow the forward procession. Many of the cellular processes involved in keratocyte movement, such as actin polymerization and membrane fluidity, are strongly affected by the physical and chemical environment (Cossins et al., 1987; Hochachka and Somero, 2002). Thus, it is to be expected that keratocyte motility and the wound healing capacity it supports would be highly sensitive to environmental temperature. The potential scale of these thermal effects is suggested by the finding that actin-based movement in mammalian fibroblast cells increased with a Q_{10} of approximately 4 between 29°C and 39°C (Hartmann-Petersen et al., 2000).

Currently, little is known about thermal effects on keratocyte motility in ectotherms and how this thermal sensitivity varies among species evolutionarily adapted to different temperatures or among conspecifics acclimated to low and high temperatures. To examine these phenomena, we studied keratocyte motility in four species of fish: two eurythermal species – a temperate goby, *Gillichthys mirabilis* (10–35°C), and a desert pupfish, *Cyprinodon salinus* (10–40°C) – and two species with narrow environmental temperature ranges – an Antarctic notothenioid, *Trematomus bernacchii* (–1.86°C), and a tropical clownfish, *Amphiprion percula* (26–30°C). In order to examine the long-term effects of acclimation as well as acute effects of experimental temperature, the first two species were acclimated to multiple temperatures within their physiological thermal range, and the stenothermal *T. bernacchii* was maintained at 5°C, the highest temperature to which this species can be acclimated (see Somero and DeVries, 1967; Hofmann et al., 2000). We describe variation in keratocyte morphology and quantify keratocyte motility in terms of cell speed and direction in relation to evolutionary, acclimatory and experimental temperatures. These data provide insights into thermal effects on nonspecific immunity and into the physiological determinants of organismal thermal tolerance limits.

Materials and methods

Experimental organisms

Gillichthys mirabilis (Cooper) (Family: Gobiidae) are found in estuarine habitats in the Gulf of California and along the California coast as far north as Tamales Bay (Eschmeyer et al., 1983). *G. mirabilis* were collected in coastal waters near Santa Barbara, CA in 1997–1999 using fish traps. *Cyprinodon salinus* (Miller) (Family: Cyprinodontidae) is found in Salt Creek, Death Valley, California (Page and Burr, 1991). Specimens were obtained from Dr G. Bernardi (University of California at Santa Cruz). *Amphiprion percula* (Lacepède) (Family: Pomacentridae) occur throughout the Western, Central and South Pacific Ocean, the Indian Ocean and the Red Sea (W. Capman, http:www.augsberg.edu/biology/aquaria). Specimens of *A. percula* were obtained from a California tropical fish store and were captive bred and raised. *Trematomus bernacchii* (Boulenger) (Family: Nototheniidae) occurs in coastal waters around Antarctica and the Antarctic Peninsula, South Shetland Islands, South Orkney Islands and Peter I Island from the shore to 695 m depth (Fischer and Hureau, 1985). *T. bernacchii* were collected in McMurdo Sound in December 1998 and January 1999 using hook and line or fish traps.

Thermal acclimation

Stocks of field-collected *G. mirabilis* were maintained at Hopkins Marine Station in a continuous-flow, open-circulation tank containing ambient water (16°C) from Monterey Bay. Individuals were acclimated to 10°C, 16°C and 25°C (±0.5°C) for a period of 4 weeks in closed recirculating tanks equipped with a flow-through heater/chiller. *C. salinus* were acclimated

for 4 weeks at temperatures of 26±0.5°C or 35±0.5°C in closed recirculating tanks in seawater taken from Monterey Bay that was adjusted to 50‰ salinity. *T. bernacchii* were studied at the US McMurdo Station. Fish were maintained in a continuous-flow, open-circulation tank in ambient water (–1.86°C) pumped in from McMurdo Sound. *T. bernacchii* acclimated to 5°C were held for 3 weeks in a closed recirculating tank heated to 5±0.5°C. *A. percula* were maintained at 26±0.5°C for 4 weeks in seawater made from Instant Ocean© (MediaCybernetics, Silver Spring, MD, USA).

Cell culture

Keratocytes were cultured in L-15 medium made with 50% filtered seawater (0.2 μm filter) and supplemented with Hepes, 5% antibiotic/antimycotic (Sigma) and 10% fetal bovine serum (FBS) (Ream, 2002). Several scales were removed, using forceps, from 2–3 fish from a single acclimation for each temperature experiment. In the case of *T. bernacchii*, scales were removed from only one fish for each experiment because of limited specimen availability. The scales were washed in medium for 15 min and then plated between untreated glass cover slips with fresh medium. Cultures of keratocytes from *G. mirabilis* acclimated to 10°C, 16°C and 25°C were maintained at 12°C, 20°C and 25°C, respectively; keratocytes from *C. salinus* and *A. percula* were maintained at 25°C; and keratocytes from *T. bernacchii* acclimated to –1.86°C and 5°C were maintained at 0°C and 4°C, respectively. Keratocytes cultured from *G. mirabilis* matured in 12 h and were viable for 36 h after maturation; keratocytes from *C. salinus* and *A. percula* matured in 6 h and were viable for 12 h after maturation; and keratocytes from *T. bernacchii* matured in 48 h and were viable for 36 h after maturation.

Supercooling of keratocytes of T. bernacchii

T. bernacchii can be supercooled to –6°C if they are previously warmed to 0°C to melt any ice crystals present in their body fluids (DeVries and Cheng, 1992). Keratocytes cultured from *T. bernacchii* (–1.86°C) were subjected to supercooling experiments in order to determine whether or not individual cells in primary culture could withstand supercooling. Glass cover slips containing keratocytes were placed in seawater at 0°C and slowly cooled to –6.0°C. Keratocytes were then slowly returned to 5°C and visualized on an Olympus light microscope to determine if they were still capable of locomotion.

Video microscopy

Cells were imaged on three different inverted light microscopes (located at the three different biological laboratories where the research was conducted) under 10×(2×), 20×(2×) or 40×(2×) magnification. The Nikon Diaphot microscope (Hopkins Marine Station) was equipped with a Peltier temperature-controlled stage, whereas the Olympus BH-2 (McMurdo Station) and Nikon Diaphot-300 (Stanford University School of Medicine) microscopes were equipped with temperature-controlled stages attached to a circulating water bath. Cell cultures, cell behavior and cell speed remained consistent across imaging conditions. Temperature was measured at the center of the surface of the cover slip in the area being imaged using a thermocouple probe. Temperature was maintained within ±0.5°C of the designated experimental temperature. The temperature of the cover slip and surrounding medium was slowly brought from room temperature to experimental temperature, taking as little as 10 min or as long as 4 h, depending on the temperature differential. Once the experimental temperature was reached, the cells were held at that temperature for a minimum of 20 min before recording data. There was no discernible difference in cell speed or behavior between cells monitored after 20 min or 4 h at experimental temperatures. Each cell was recorded for 15 min and video frames were captured every 15 s, resulting in a total of 61 frames per cell. Depending on the speed and path of the cell, many cells crawled out of the field of view. When this occurred, the time lapse was paused, the stage was moved in order to replace the cell in the field of view, and the time lapse was then continued. Each pause resulted in a temporal and spatial discontinuity that was accounted for in the analysis but which often reduced the total number of useful frames per cell. A total of 20 cells from a minimum of two cultures were imaged at each experimental temperature. No more than 12 cells per culture were imaged for a given temperature condition.

Image analysis

Movies of keratocytes imaged on the Nikon (Hopkins Marine Station) or Olympus microscopes were acquired using a Silicon Intensifier Target (SIT) camera (Nikon; Hopkins Marine Station) or chilled charge-coupled device (CCD) camera (Olympus) attached to a VCR and recorded in real time to VHS. Images were pulled from the tapes every 15 s using ImagePro Plus® data analysis software (MediaCybernetics). Time-lapse video was acquired digitally for keratocytes imaged on the Nikon (Stanford University Medical School) microscope using an intensified charge-coupled device (ICCD) camera (GenII Sys/CCD-c72) with the shutter driver and acquisition capabilities of MetaMorph® image analysis software (Universal Imaging Corporation™; Downingtown, PA, USA). Frames were captured every 15 s and compiled into time-lapse movies. Keratocytes were tracked by hand using a mouse and the 'point-and-click' method utilized by both software packages, yielding discrete (x,y) coordinates for the keratocyte at every 15 s interval. The cell body and leading edge of the lamellipodium were tracked for >60 cells and no discernible difference was found between the two tracking methods. The cell body was chosen for simplicity and all cells used in this analysis were tracked with that method.

Analysis of cell velocity

The speed of a cell was determined as the instantaneous speed over 15 s for each continuous 15 s interval. All instantaneous speeds measured for all cells at a given experimental condition were placed into bins and the frequency

of occurrence was determined for each bin. The mean and median instantaneous velocities over 15 s were also determined for each cell. Autocorrelation analysis of instantaneous speed over time was used to determine the degree of memory associated with speed (i.e. are instantaneous speed measurements independent or related to previous speeds?) where the speed autocorrelation over k time intervals is given by:

$$\sum_{i=1}^{N-k} (u_{i+k} - \bar{u})(u_i - \bar{u})\{1/[\text{S.D.}(N-k)]\},$$

where

$$\text{S.D.} = \sum_{i=1}^{N} (u_i - \bar{u})^2 \times (1/N),$$

where S.D. is standard deviation, u_i is speed measured over interval i, u_{i+k} is speed measured k intervals later, k is offset instantaneous speed, $\bar{u}$ is mean speed and N is sample size. Autocorrelation values range from +1 to –1. Speed autocorrelations for moving cells typically decay to 0 over increasing time intervals ranging from a few seconds to a few minutes. Some cell types demonstrate periodic oscillations in speed resulting in oscillating autocorrelation functions.

Keratocyte paths were characterized with respect to directional movement. Directional movement can be visualized by plotting the trajectory of each cell as (x,y) coordinates. Each trajectory begins at the origin and is rotated so that the first step taken by each cell is in the same direction (x=0). A matrix using non-overlapping intervals was used to determine all possible path lengths for this analysis. Additionally, the magnitude of the turns made by keratocytes can be examined by plotting the mean cos (θ), or turning angle, against the path length traveled by each cell for each pair of (x,y) coordinates. This was done using non-overlapping intervals for all possible path lengths. This method does not reveal directionality, because it takes into account turning magnitude only. Mathematical formulas used in these analyses can be found in Ream (2002).

Statistical analysis

Calculation of mean speed, median speed, standard deviation and standard error for each cell was performed using Microsoft Excel®. The statistical analysis package in Sigma Plot® version 5.0 was used to perform Student's t-tests to determine whether or not the increase in keratocyte speed between experimental temperatures was statistically significant. The Microsoft Excel® statistical software package was employed to run two-way analyses of variance (ANOVAs) between experimental temperatures within each acclimation to determine if thermal acclimation had an effect on speed.

Results

Keratocyte morphology

Keratocytes exhibited wide variation in size, shape, pattern of shape change and lamellipodial ruffling, and this morphological variation differed among species. Fig. 1 illustrates the range of morphologies that were observed. Keratocytes from all species underwent some degree of change in shape that was consistent with large-scale directional changes, which were frequently coupled to changes in the leading edge designation. The amount of time spent by a cell determining which edge of the cell became the new leading edge, along with the frequency of morphological changes involved, varied among species.

Keratocytes from *G. mirabilis* exhibited the greatest size variation. Within a single culture, cells of average size (≈35 μm wide along the long axis) could be found along with cells as large as ≈80 μm and as small as ≈15 μm in width. For the other species, size variation within a species was only approximately ±5 μm. Regardless of their size, *G. mirabilis* keratocytes displayed the characteristic 'canoe shape' first described by Goodrich (1924), which denotes an elongated, elliptical cell body with a smooth-edged, narrow lamellipodium running along one side of the cell body and smoothly curving around each end (Fig. 1A). Some degree of ruffling was apparent over time and usually occurred at the

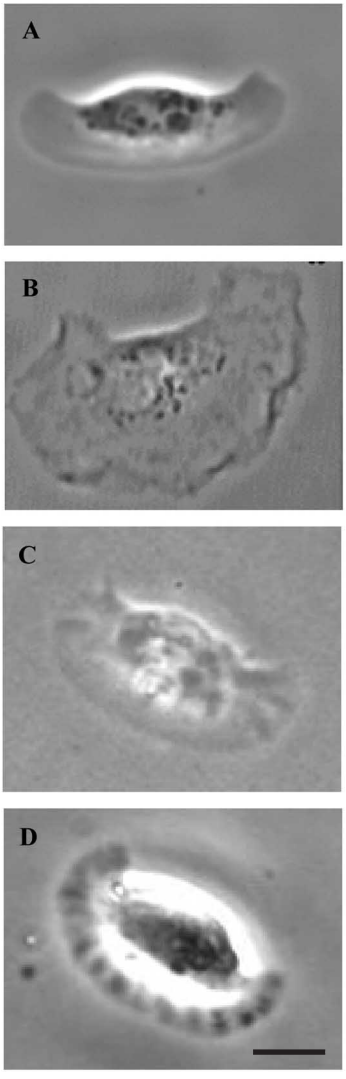

Fig. 1. Phase-contrast images of keratocytes from *G. mirabilis* display the range of variation observed in keratocyte morphology. Morphological features include: (A) stereotypical 'canoe shape', (B) small ruffling at the leading edge, (C) ruffling at the edges of the cell and (D) large, phase-dense ruffles in the forward third of the lamellipodium. Scale bar, 10 μm.

leading edge of the cell, although the edges of the lamellipodium were generally smooth. Shape-changing events in *G. mirabilis* keratocytes had durations of 1–2 min, occurred in fewer than half of the cells and involved few lamellipodial rearrangements.

Keratocytes of *C. salinus* were ≈35 μm wide and varied little in size. The keratocytes were less canoe-shaped in appearance due to a lengthening in the lamellipodia between the cell body and the leading edge, which gave the cells a fatter appearance. Additionally, the lamellipodia of these cells ruffled >50% of the time. These ruffles were almost always in the first third of the lamellipodium at the leading edge of the cell and appeared as large, rapidly changing, phase-dense regions rather than small dark creases. Shape changes in *C. salinus* keratocytes occurred in slightly fewer than half of the cells, lasted approximately 2–4 min and involved more lamellipodial rearrangements than in cells of *G. mirabilis* and *A. percula*.

Keratocytes of *A. percula* were ≈35 μm wide along the long axis and had the stereotypical canoe shape. The leading edges of their lamellipodia were gently rounded, and most ruffling occurred at the ends of the lamellipodia rather than at the leading edge. Shape changing events in *A. percula* keratocytes had durations of 1–2 min, occurred in less than half of the cells undergoing shape changes and few had lamellipodial rearrangements.

T. bernacchii keratocytes also displayed the characteristic canoe shape and had minimal variation in size (≈35 μm wide along the long axis). The lamellipodia always appeared smooth and any ruffling was in the form of small creases in the lamellipodia at the edges of the cell body. The most notable feature of *T. bernacchii* keratocytes was their extremely slow rate of locomotion. Locomotion, lamellipodial ruffling or any manner of shape change was only detectable using time-lapse microscopy, in contrast to the cells of other species where dynamic events were often apparent to the eye. Shape changes occurred in a little over half of the cells, lasted approximately 4–5 min and involved a large number of lamellipodial rearrangements.

Keratocyte motility can be characterized in terms of a velocity comprising a magnitude (speed) and a complex directional component (Figs 2, 3). The directional component can be further broken down into persistence, or movement in a single direction, and turning behavior, namely the size of the angles made by a cell as it turns. Both components of velocity as well as the thermal range over which motility was observed, varied among species and, in some cases, with acclimation history.

The temperature ranges over which keratocyte motility occurred reflected the evolutionary adaptation temperatures of the species (Fig. 4). Keratocytes of *T. bernacchii* maintained at the normal McMurdo Sound temperature (–1.86°C) or acclimated to 5°C were functional over the full range of measurement temperatures (5–20°C). Keratocytes from *T. bernacchii* (–1.86°C) that were supercooled to –6.0°C for 10 min had normal motility when they were returned to 5°C. Keratocytes cultured from *G. mirabilis* acclimated to 10°C, 16°C and 25°C were able to function at 10–35°C. The keratocytes isolated from *A. percula* maintained at 26°C were also functional at 10–35°C. Keratocytes from *C. salinus* acclimated to 26°C and 35°C were functional at 10–40°C and 5–40°C, respectively. In all cases, the thermal range of the keratocytes was at least as broad as the thermal range of the fishes' habitats, and, for the two stenothermal species (*T. bernacchii* and *A. percula*), the thermal range of keratocyte motility was much greater than the environmental temperature range of the fish.

Thermal effects on keratocyte speed

Mean speed of keratocyte movement varied among species and as a function of experimental temperature (Fig. 4). Autocorrelation analysis of instantaneous speeds measured over 15 min varied from cell to cell and showed no strong correlation and no oscillation in speed for any species examined (Table 1), indicating that each instantaneous speed is discrete and is not affected by previous cell speed.

G. mirabilis keratocyte motility significantly increased in speed with increasing temperature from 5°C to 25°C (P<0.001 in all cases) with a Q_{10} of ≈2.5 (Fig. 4A). At 25°C, mean speed reached a maximum and then decreased at higher temperatures. Variation in instantaneous speed also increased with increasing measurement temperature between 5°C and 35°C. Although the mean speed of keratocytes from individuals held at all three acclimation temperatures exhibited the same response to experimental temperature (P=0.53), mean speed varied with acclimation temperature, with cells from the 10°C group most commonly having the slowest speeds (Fig. 4A). At low experimental temperatures of 5–15°C, keratocytes from 25°C-acclimated fish moved more rapidly than keratocytes from the two cold acclimations (16°C and 10°C). Conversely, cells from cold-acclimated fish (10°C) moved more slowly at warmer temperatures (20–35°C) than those from the two warmer acclimations (16°C and 25°C). The maximal speed of ≈45 μm min^{-1} was reached by keratocytes from the 16°C acclimation. The mean speed for all the acclimation groups at

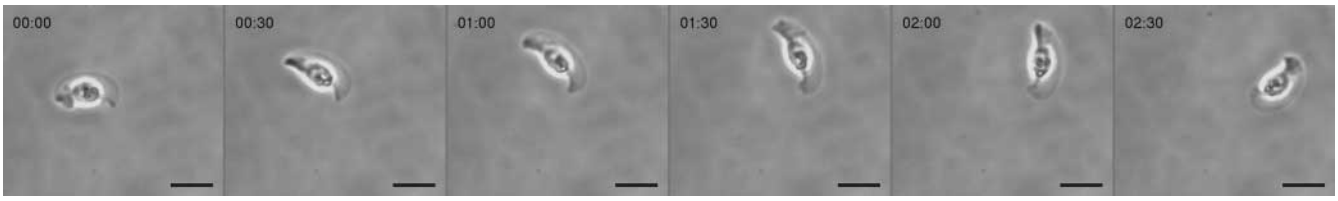

Fig. 2. Time-lapse picture of crawling keratocyte cultured from *G. mirabilis* at 2(40×). Scale bar, 15 μm. Images were taken 30 s apart.

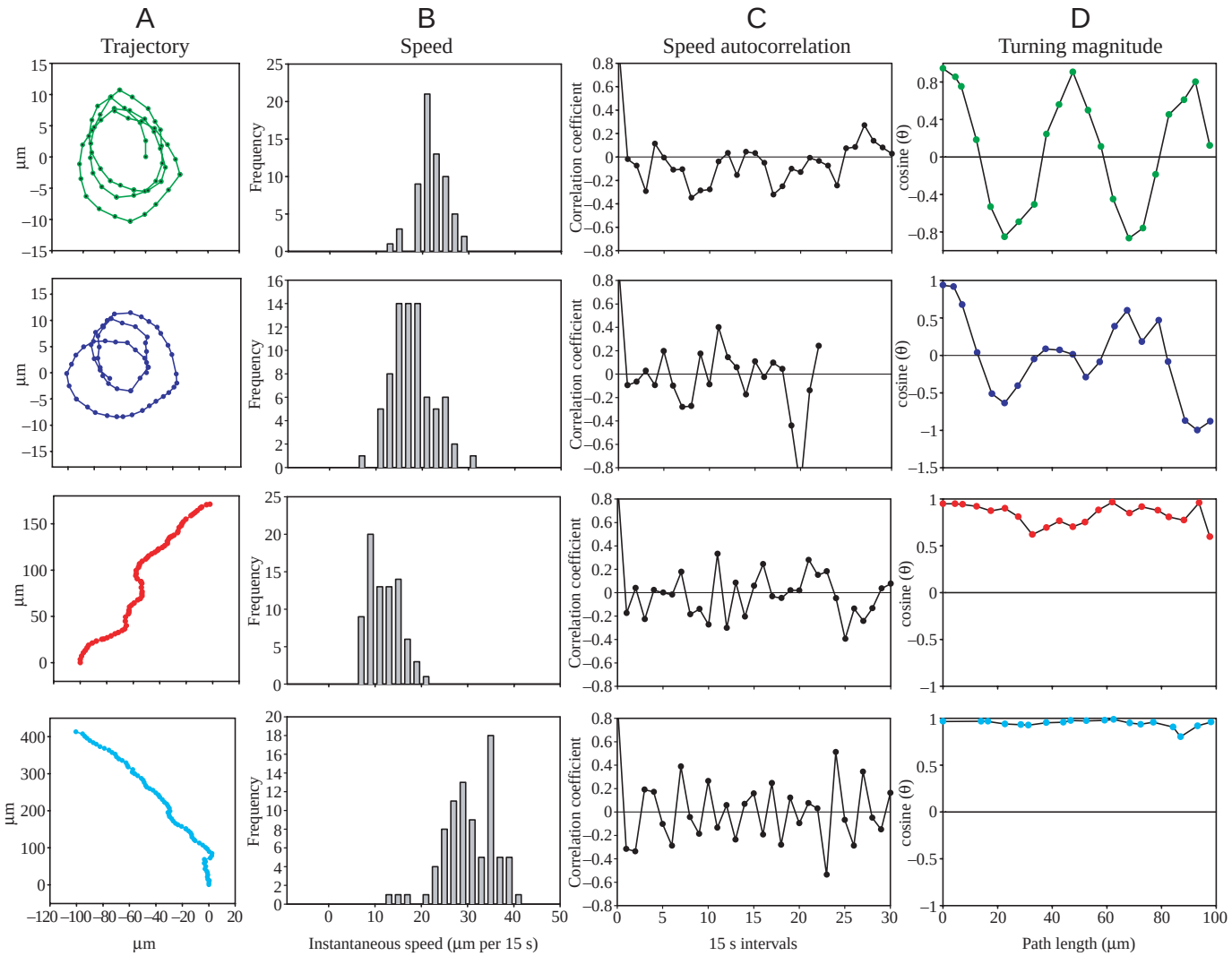

Fig. 3. The velocity of each cell was analyzed in regard to speed and trajectory. Examples show four types of analysis for four cells cultured from *G. mirabilis*, two moving in straight lines and two moving in circles: (A) a trajectory plot of (*x*,*y*) coordinates of the positions of four cells over 20 min, beginning at the origin and rotated such that the first step is along the *y*-axis (*x*=0); (B) speed histograms of the frequency of instantaneous cell speeds; (C) autocorrelation analysis of cell speed shows memory (correlation) and oscillation; and (D) cosine analysis shows the magnitude of turning angles (θ) made by the cell over time.

15°C, the experimental temperature that was closest to the habitat temperatures of the population used, was 11.4 μm min^{-1}.

C. salinus keratocyte speed increased with increasing temperature from 5°C to 35°C, with a Q_{10} of 2.0, and decreased between 35°C and 40°C. The speeds are statistically different at each temperature (P<0.001 in all cases) except for cells of individuals acclimated to 35°C, which moved at the same rate at 25°C and 30°C. The variation in speed also increased with temperature. *C. salinus* keratocytes from both acclimation temperatures exhibited the same response to experimental temperature (P=0.46), although mean speed varied between acclimation groups at several measurement temperatures. Keratocytes from both thermal acclimations did not vary in speed at low (10–15°C) and high (35–40°C) experimental temperatures but crawled at statistically different speeds at the middle experimental temperatures (20–30°C; Fig. 4B). At 20°C, the cold-acclimated cells moved faster, but at 25–30°C the warm-acclimated cells were the fastest moving. The mean speed for all the acclimation groups at 30°C, a common physiological temperature for *C. salinus*, was 13.8 μm min^{-1} (Fig. 5). The maximal instantaneous speed was ≈45 μm min^{-1} at 35°C. Both the mean speed at physiological temperature and the maximal speed were very similar to those of keratocytes from *G. mirabilis.*

Motility of keratocytes of *A. percula* significantly increased in speed as temperature increased from 10°C to 35°C (P<0.001), except between 15°C and 20°C, at which temperatures the keratocytes exhibited similar speeds (P=0.095; Fig. 4C). The overall Q_{10} was ≈2.0. The maximal instantaneous speed measured was ≈45 μm min^{-1}. Variation in instantaneous speed also increased with increasing temperature

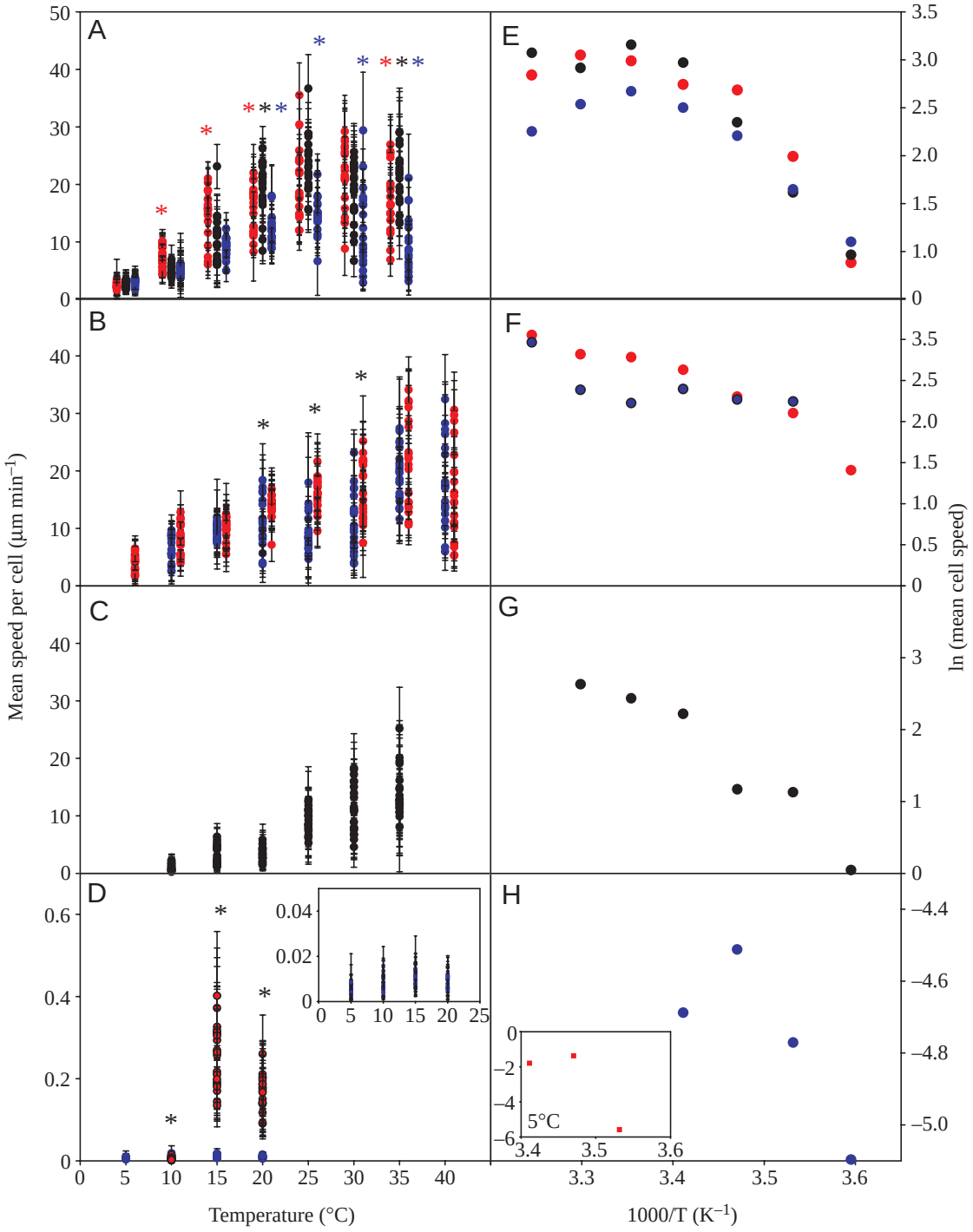

Fig. 4. The mean cell speed ± S.D. for 20 cells at each experimental temperature. Cells cultured from a thermal acclimation group denoted by an asterisk move at a mean speed significantly different from the cells of other thermal acclimations at that experimental temperature. (A) Keratocytes from *G. mirabilis* acclimated to 10°C (blue), 16°C (black) and 25°C (red); (B) keratocytes from *C. salinus* acclimated to 26°C (blue) and 35°C (red); (C) keratocytes from *A. percula* acclimated to 26°C (black); and (D) keratocytes from *T. bernacchii* acclimated to –1.86°C (blue; inset) and 5°C (red; main figure) move at speeds 10–100× slower than other cells. (E–H) Arrhenius plots of 1000/*T* *vs*. ln(speed) for cells cultured from *G. mirabilis* (E), *C. salinus* (F), *A. percula* (G) and *T. bernacchii* (H). Acclimation groups are delineated by the corresponding color codes in panels A–D.

between 5°C and 15°C and between 20°C and 35°C. The mean speed at 30°C, a common physiological temperature, was 10.8 μm min^{-1} (Fig. 5).

Keratocytes cultured from *T. bernacchii* held at –1.86°C also exhibited temperature-dependent rates of motility (Fig. 4D, inset). Keratocyte speeds are statistically different

Table 1. *Autocorrelation analysis of speed reveals that the number of cells showing oscillation in speed or memory (correlation) is species-specific and varies with temperature acclimation and experimental temperature*

Organism	Experimental temperature (°C)	Autocorrelation	
		Oscillatory	Correlation
Gillichthys mirabilis			
10°C acclimation	5	0	1
	10	3	1
	15	6	0
	20	9	1
	25	6	1
	30	3	3
	35	4	3
16°C acclimation	5	2	2
	10	5	1
	15	8	0
	20	8	0
	25	1	2
	30	4	2
	35	2	0
25°C acclimation	5	1	0
	10	5	0
	15	3	0
	20	3	0
	25	4	0
	30	4	2
	35	3	2
Amphiprion percula			
26°C acclimation	10	0	1
	15	0	1
	20	0	6
	25	4	2
	30	2	5
	35	6	4
Cyprinodon salinus			
26°C acclimation	10	0	0
	15	5	0
	20	5	1
	25	4	1
	30	1	0
	35	1	0
	40	2	1
35°C acclimation	5	1	1
	10	3	1
	15	2	2
	20	2	2
	25	6	0
	30	3	0
	35	3	2
	40	4	1
Trematomus bernacchii			
–1.86°C acclimation	5	0	0
	10	6	11
	15	6	10
	20	3	6
5°C acclimation	10	2	0
	15	1	0
	20	6	0

(P<0.05) between the experimental temperatures of 5°C, 10°C and 15°C; however, keratocytes moved at the same speed at 15°C and 20°C (P=0.068). The maximal instantaneous speed measured was 0.046 μm min^{-1}, a small fraction of the maximal velocity of the other species' keratocytes. The mean speed at 5°C, the experimental temperature closest to physiological (–1.86°C) was 0.008 μm min^{-1}. Thus, no temperature compensation of rate of motility was found for keratocytes of *T. bernacchii* (Fig. 5). Although the mean speed of keratocytes from *T. bernacchii* held at both acclimation temperatures exhibited the same response to experimental temperature (P=0.63), acclimation groups were statistically different (P<0.01) from each other at each experimental temperature. The strong acclimation effect on the speed of keratocytes from animals acclimated to 5°C is most obvious at the experimental temperatures of 15°C and 20°C, where speed was 10–20-fold greater than in the –1.86°C-acclimated cells. Variability in speeds was enormous, with speeds in the range of 0.065–1.05 μm min^{-1}. Keratocyte speeds were statistically different (P<0.01) at each experimental temperature.

Thermal effects on keratocyte trajectories

Two types of analysis were used to quantitatively describe the directional component inherent in cell velocity. The first type of analysis plots the trajectory of each cell, beginning at the origin and oriented such that the first step taken by each cell is along the *x*-axis. Trajectories were compared at all experimental temperatures; however, general trends can be summarized by comparing trajectories at 10°C and 20°C (Fig. 6). This analysis made it possible to visualize path shape and to determine the degree of persistence in a single direction. There were cells taking very straight and persistent trajectories at all experimental temperatures and acclimations; however, the number of individual cells exhibiting this type of directional movement varied with experimental temperature and acclimation. The second type of analysis, plotting the cos (θ) of vectors tangential to the cell trajectory *versus* path length traveled between each pair of vectors, revealed turning magnitudes in highly persistent cells that were not obvious in the trajectory analysis (Fig. 7).

Keratocytes exhibited a range of turning behaviors that varied with both experimental and acclimation temperatures. At lower experimental temperatures, cells from all species traveled in persistently straight trajectories, regardless of acclimation history. Keratocytes from *T. bernacchii* had the greatest persistence at all experimental temperatures of any species examined, and persistence was not affected by experimental temperature. For all non-Antarctic species, as experimental temperature increased, persistence in a single direction decreased and turning associated with random walk behavior increased. Interestingly, persistent circular trajectories appear with increasing experimental temperature in the case of keratocytes cultured from *G. mirabilis* and *C. salinus*. *G. mirabilis* keratocytes took circular paths only at experimental temperatures of ≥20°C. Additionally, keratocytes from individuals acclimated to 10°C were more likely to have

circular trajectories than those acclimated to 16°C, while fish acclimated to 25°C had the greatest number of cells with circular trajectories. *C. salinus* keratocytes were less persistent at lower experimental temperatures, and at higher experimental temperatures they exhibited turns of larger radii compared with *G. mirabilis* keratocytes, with few *C. salinus* cells making completely circular paths at experimental temperatures below 35°C. Furthermore, trajectories varied with acclimation temperature; keratocytes from cold-acclimated fish (26°C) were less persistent with more random walks at experimental temperatures below 20°C and showed a greater incidence of curved and circular paths at temperatures greater than 20°C than cells from the warm-acclimated group.

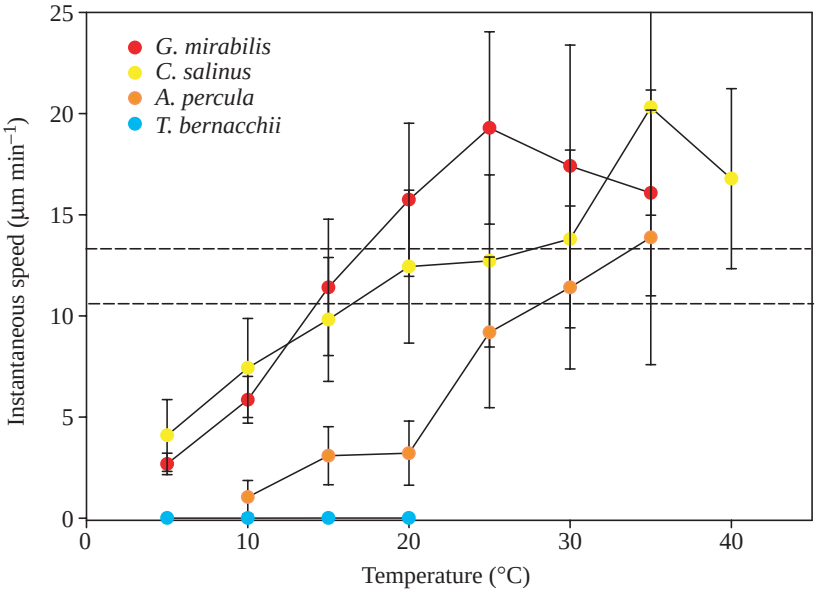

Fig. 5. Mean keratocyte speed as a function of experimental temperature. Speed is conserved at physiological temperatures for the three non-Antarctic species, as indicated by the zone bounded by the two horizontal lines.

Analysis of the cos (θ) *versus* path length reveals that the turning angles between two adjacent video time-lapse intervals (path lengths ≤20 μm) are less sharp than the turning angles between non-adjacent intervals spaced further apart, which shows that the turning angle increases with increased path length [average cos (θ)=0.1 to 0.9, depending on path length]. Thermal acclimation affected the magnitude of directional changes taken by cells of all species examined. Keratocytes from *G. mirabilis* and *C. salinus* acclimated to 10°C and 26°C, respectively, displayed greater turning angles than the cells from warmer acclimations. *G. mirabilis* cells acclimated to 16°C took the straightest paths at 20°C, and thermal acclimation had the strongest effect on turning magnitude at 20°C. The turning magnitudes of keratocytes cultured from *C. salinus* exhibited greater turning magnitude at higher temperatures, and cells acclimated to 35°C took the straightest paths at 10°C. *A. percula* keratocytes made larger angle turns than the other non-Antarctic species, and their turning behavior was statistically the same (P>0.05) at every experimental temperature. Thermal acclimation affected turning behavior in *T. bernacchii* keratocytes at path lengths greater than 2 μm, with –1.86°C-acclimated cells making larger turns at low temperatures and 5°C-acclimated cells making larger turns at high temperatures.

Discussion

This is the first comparative description of the effects of temperature on fish keratocyte motility. A primary conclusion from this study is that the speed and persistence of movement of teleost keratocytes are highly variable and are complex functions of evolutionary, acclimatory and measurement temperatures. The effect of measurement temperature on rate of motility yielded Q_{10} values of ≈2 for most species and acclimation groups, which suggests that the biochemical processes that are responsible for actin-based motility have relatively consistent thermal sensitivities across species. However, the absolute rates of motility differed significantly among species. Keratocytes of the two eurythermal species, *G. mirabilis* and *C. salinus*, were capable of crawling the fastest, with maximal instantaneous rates of ≈40–45 μm min^{-1}. It is possible that there is some limit to keratocyte motility indicated by this maximal speed that might be reflected in an underlying cellular process governing motility rates.

For all species except the Antarctic notothenioid *T. bernacchii*, rates of movement reflected compensation for temperature (Fig. 5). Thus, at common habitat temperatures, the keratocytes of *G. mirabilis*, *C. salinus* and *A. percula* had mean speeds of 11.4 μm min^{-1} (15°C), 13.8 μm min^{-1} (30°C) and 10.8 μm min^{-1} (30°C), respectively. Although we did not directly examine the process of wound healing in this study, we conjecture that conservation of a certain range of motility rates is advantageous for ensuring that the role of keratocytes in the wound-healing process is retained across adaptation temperatures. Therefore, it is likely that keratocytes moving at 11–14 μm min^{-1} are capable of adequate migration to be effective in wound closure for prevention of infection. Slower rates may not be as effective and more rapid locomotion might be too costly, metabolically, and not yield a sufficient increase in protective value.

Although temperature compensation of keratocyte motility was observed in comparisons among the non-Antarctic species, none of the species studied showed acclimatory temperature compensation of motility. The lack of acclimatory temperature compensation suggests that seasonal variation in wound closure behavior may exist. However, until it is known how pathogen activity varies with temperature, the potential importance, if any, of this seasonal change cannot be established.

The very low speeds recorded for keratocytes of *T.*

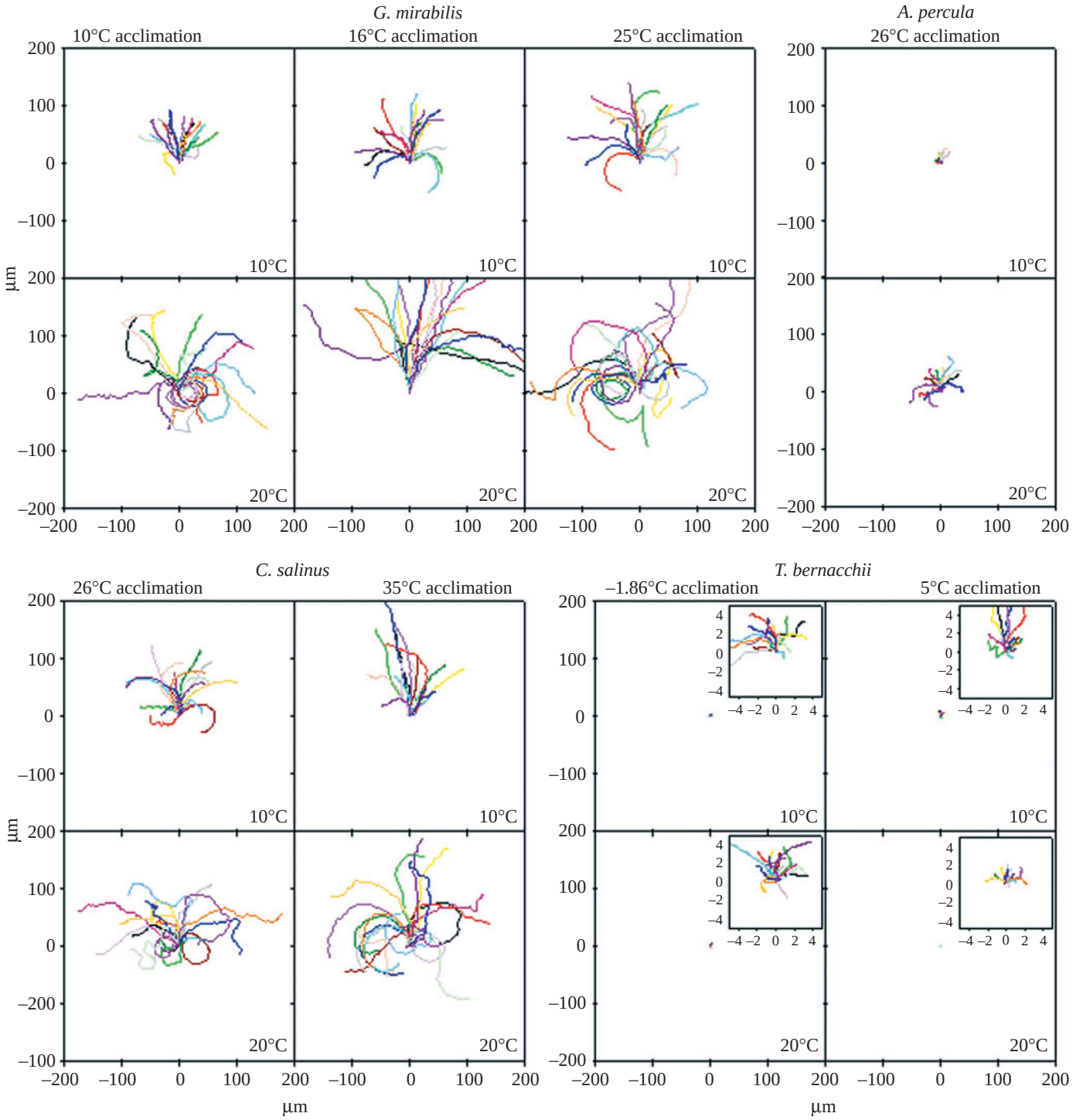

Fig. 6. Cell centroid positions were tracked over a total time of 15 min at 15 s intervals for 20–21 cells at each experimental condition. For population comparisons, each cell's path was plotted as a separate colored line beginning at the origin and reoriented so that the first segment of any trajectory is along the *y*-axis (x=0). Trajectories of each cell measured are shown at 10°C and 20°C for all acclimation groups of all species. Systematic variations in both speed and turning behavior are apparent.

bernacchii are paradoxical in the context of the proposed importance of conservation of wound-healing behavior. Keratocytes of the Antarctic fish moved at rates that were less than one percent those of the other species at their physiological temperatures. We do not interpret these extremely low rates of movement to be an indication of a 'failure' to cold-adapt, because Antarctic notothenioids have a pronounced level of cold-adapted metabolic and enzymatic activity in certain tissues (Somero et al., 1968; Kawall et al., 2002) and there is no *a priori* basis for assuming that cold

adaptation is precluded in any particular cell type. Thus, the biological significance, if any, of the extremely slow rates of movement of the keratocytes of *T. bernacchii* is not apparent. Likewise, the significance of the increased mobility of keratocytes from 5°C-acclimated *T. bernacchii* is unclear. Because 5°C is several degrees above the highest temperatures that this species experiences (and likely has experienced for 10–15 million years), the effects of acclimation to 5°C could represent a pathological condition.

The range of temperatures over which keratocyte motility was observed and the thermal optima of keratocyte speed reflected both evolutionary and acclimation temperatures (Fig. 4). The ability of keratocytes of *T. bernacchii* to survive 10 min of supercooling at –6°C and display apparently normal rates of movement at 5°C after the bout of supercooling supports the observation of DeVries and Cheng (1992) that *T. bernacchii* can withstand these very low temperatures as long as ice crystals are absent in the internal fluids and medium. The ability of keratocytes of *T. bernacchii* to sustain motility at a temperature of 20°C, which is approximately 15°C above the upper lethal temperature of this species (Somero and DeVries, 1967; Hofmann et al., 2000), indicates that the mechanisms underlying acute thermal death may involve complex physiological functions (for example, failure of synaptic transmission) rather than the survival of individual cells (Somero, 1996; Hochachka and Somero, 2002).

In only one instance did thermal acclimation affect the functional thermal range of cultured keratocytes: cells cultured from *C. salinus* acclimated at 26°C did not survive at 5°C, whereas cells from fish acclimated to the warmer 35°C did. This suggests a potential thermal stress for *C. salinus* acclimated at 26°C, which was responsible for decreasing the range of cellular thermal tolerance.

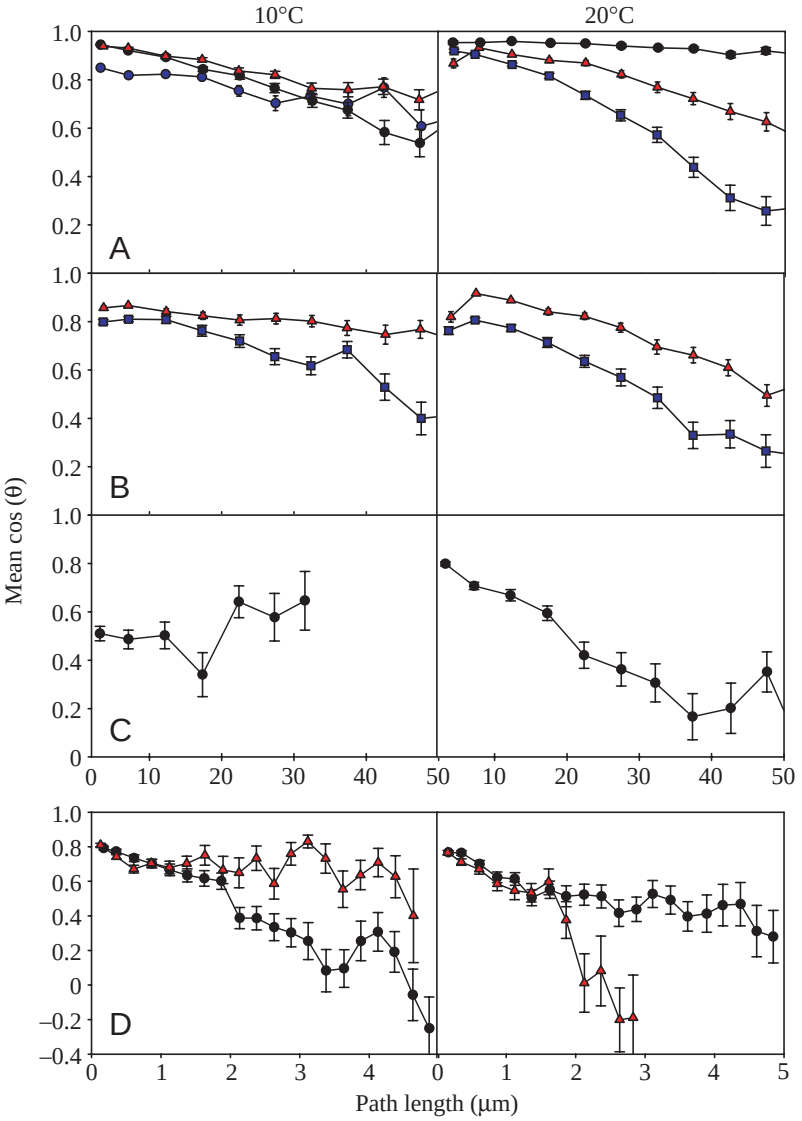

Fig. 7. Cos (θ) *versus* path length is plotted at 10°C and 20°C for cells from each species at each thermal acclimation. As turning magnitude increases, cosine values begin to approach zero. (A) Keratocytes from *G. mirabilis* acclimated to 10°C (blue), 16°C (black) and 25°C (red); (B) keratocytes from *C. salinus* acclimated to 26°C (blue) and 35°C (red); (C) keratocytes from *A. percula* acclimated to 26°C (black); and (D) keratocytes from *T. bernacchii* acclimated to –1.86°C (black) and 4°C (red).

Effects of experimental temperature were noted on the persistence and directionality of keratocyte movement, with persistently straight trajectories giving way to random walks and persistent circular trajectories as experimental temperature increased. Thermal acclimation also affected turning behavior, with cells from cold-acclimated specimens generally making turns of greater magnitude. Although *G. mirabilis*, *C. salinus* (35°C acclimation) and *A. percula* keratocytes appear very persistent at 5°C and 10°C in the path length *versus* displacement analysis, the cos (θ) analysis reveals that they are making large turns and must be moving in an oscillatory, yet persistent, manner. One of the most noteworthy results is that thermal acclimation does not affect how the keratocytes' mean speeds change with experimental temperature but does influence how the experimental temperature affects directional movement, in terms of persistence and turning angle, in *G. mirabilis* and *C. salinus*. Speed memory and oscillation are also affected by temperature acclimation.

Collectively, these results indicate that there is more than one temperature-sensitive mechanism governing cell motility. The rate-limiting process(es) responsible for speed is distinct from the mechanism(s) underlying directionality and persistence, and each mechanism is affected differently by temperature. Manipulation of temperature may allow a decoupling of these mechanisms and permit their individual study, allowing a detailed analysis of the effects of temperature on motility within and between species to be made. For

example, the polymerization and depolymerization of cytoskeletal tubulin subunits in Antarctic fish occur at a reduced rate compared with those in mammals (Detrich et al., 2000). Similar differences in orthologous biochemical systems related to keratocyte motility may be present in species from different thermal niches. Examining the thermal responses of such processes as actin polymerization, membrane fluidity or binding/release of adhesion proteins may lead to insights into the mechanisms that underlie the thermal sensitivities of keratocyte speed and directional behavior. However, we have previously shown that there are only small, conservative changes in the sequences of α-actins from vertebrates adapted to habitat temperatures ranging over 42°C, and α-actins of Antarctic notothenioids exhibit no apparent adaptation at the level of primary structure (Ream, 2002). Therefore, temperature-dependent variation in motility is probably not attributable to intrinsic variation between actin homologs.

One fundamental question that remains for future analysis is the interplay that may exist between speed and directional behavior in wound healing. It is possible that the variation in trajectory in response to temperature acclimation and experimental temperature has a functional significance, much as we conjectured for the conservation of speed *per se*, because memory and oscillation are present in a significant number of cells only at physiological temperatures.

In this study, we attempted to isolate external variables and focus solely on thermal effects; however, *in situ* wound healing occurs in the presence of numerous signaling molecules that are known to influence migration in other cell types. Obviously, temperature will affect not only the rate of diffusion of these molecules but also the transduction of chemical signals. Further examination of keratocyte motility during wound healing *in situ* may provide information on the interplay of chemical signals and/or an electrical gradient (established across the wound) on the temperature-dependent speed and directionality of these cells. Our initial descriptions of thermal effects on keratocyte function thus open up a number of avenues of study in cell biology and evolutionary physiology that can be addressed using this promising experimental system.

We acknowledge support from National Science Foundation grants IBN-0133184 to G.N.S. and OPP-9812707 to Dr Donal Manahan in support of the studies in Antarctica conducted during the McMurdo Antarctic Biology Course. We thank Dr Manahan and the staff of the Crary Laboratory for their efforts on behalf of this research. J.A.T. was supported by a David and Lucile Packard Fellowship in Science and Engineering.

References

Baudoy, A. M., Danton, M. and Merle, G. (1980). Virémie printanière de la carpe: étude expérimentale de l'infection évoluant è différentes températures. *Ann. Virol.* **131E**, 479-488.

Bly, J. E. and Clem, L. W. (1991). Temperature-mediated processes in teleost immunity: *in vitro* immunosuppression induced by *in vivo* low temperature in channel catfish. *Vet. Immunol. Immunopathol.* **28**, 365-377.

Bly, J. E. and Clem, L. W. (1992). Temperature and teleost immune functions. *Fish Shellfish Immunol.* **2**, 159-171.

Collazos, M. E., Ortega, E. and Barriga, C. (1994). Effect of temperature on the immune system of a cyprinid fish (*Tinca tinca, L.*). Blood phagocyte function at low temperature. *Fish Shellfish Immunol.* **4**, 231-238.

Cossins, A. R., Behan-Martin, M., Jones, G. and Bowler, K. (1987). Lipid–protein interactions in the adaptive regulation of membrane function. *Biochem. Soc. Trans.* **15**, 77-81.

Detrich, H. W., III, Parker, S. K., Williams, R. C., Jr, Nogales, E. and Downing, K. H. (2000). Cold adaptation of microtubule assembly and dynamics. Structural interpretation of primary sequence changes present in the alpha- and beta-tubulins of Antarctic fishes. *J. Biol. Chem.* **275**, 37038-37047.

DeVries, A. L. and Cheng, C.-H. C. (1992). The role of antifreeze glycopeptides and peptides in the survival of cold-water fishes. In *Water and Life* (ed. G. N. Somero, C. B. Osmond and C. L. Bolis), pp. 301-315. Berlin: Springer-Verlag.

Dexiang, C. and Ainsworth, A. J. (1991). Effect of temperature on the immune system of channel catfish (*Ictalurus punctatus*). II. Adaptation of anterior kidney phagocytes to 10°C. *Comp. Biochem. Physiol. A* **100**, 913-918.

Ellsaesser, C. F. and Clem, L. W. (1986). Haematological and immunological changes in channel catfish stressed by handling and transport. *J. Fish Biol.* **28**, 511-521.

Eschmeyer, W. N., Herald, E. S. and Hamann, H. (1983). *A Field Guide to Pacific Coast Fishes of North America*. Boston: Houghton Mifflin.

Euteneuer, U. and Schliwa, M. (1984). Persistent directional motility of cells and cytoplasmic fragments in the absence of microtubules. *Nature* **310**, 58-61.

Fischer, W. and Hureau, J. C. (1985). *FAO Species Identification Sheets for Fishery Purposes: Southern Ocean (Fishing Areas 48, 58 and 88) (CCAMLR Convention Area)*. Rome: Food and Agriculture Organization of the United Nations.

Goodrich, H. B. (1924). Cell behavior in tissue cultures. *Biol. Bull.* **46**, 252-262.

Hartmann-Petersen, R., Walmod, P. S., Berezin, A. V. and Bock, E. (2000). Individual cell motility studied by time-lapse video recording: influence of experimental conditions. *Cytometry* **40**, 260-270.

Hochachka, P. and Somero, G. N. (2002). *Biochemical Adaptation: Mechanism and Process in Physiological Evolution.* New York: Oxford University Press.

Hofmann, G. E., Buckley, B. A., Airaksinen, S., Keen, J. E. and Somero, G. N. (2000). Heat-shock protein expression is absent in the Antarctic fish *Trematomus bernacchii* (Family Nototheniidae). *J. Exp. Biol.* **203**, 2331-2339.

Kawall, H. G., Torres, J. J., Sidell, B. D. and Somero, G. N. (2002). Metabolic cold adaptation in Antarctic fishes: evidence from enzymatic activities of brain. *Mar. Biol.* **140**, 279-286.

Le Morvan, C., Clerton, P., Deschaux, P. and Troutaud, D. (1997). Effects of environmental temperature on macrophage activities in carp. *Fish Shellfish Immunol.* **7**, 209-212.

Le Morvan, C., Deschaux, P. and Troutaud, D. (1996). Effects and mechanisms of environmental temperature on carp (*Cyprinus carpio*) anti-DNP antibody response and nonspecific cytotoxic cell activity: a kinetic study. *Dev. Comp. Immunol.* **20**, 331-340.

Le Morvan, C., Troutaud, D. and Deschaux, P. (1995). Effects of temperature on carp leukocyte mitogen-induced proliferation and non-specific cytotoxic activity. *Dev. Comp. Immunol.* **19**, 87-95.

Le Morvan, C., Troutaud, D. and Deschaux, P. (1998). Differential effects of temperature on specific and nonspecific immune defenses in fish. *J. Exp. Biol.* **201**, 165-168.

Page, L. M. and Burr, B. M. (1991). *A Field Guide to Freshwater Fishes of North America North of Mexico*. Boston: Houghton Mifflin.

Radice, G. P. (1980a). The spreading of epithelial cells during wound closure in *Xenopus* larvae. *Dev. Biol.* **76**, 26-46.

Radice, G. P. (1980b). Locomotion and cell-substratum contacts of *Xenopus* epidermal cells in vitro and in situ. *J. Cell Sci.* **44**, 201-223.

Ream, R. A. (2002). The temperature biology of vertebrate actins and the actin-based motility of fish keratoytes. PhD thesis, Stanford University, Palo Alto, CA, USA.

Ream, R. A., Johns, G. C. and Somero, G. N. (2003). Base compositions of genes encoding alpha-actin and lactate dehydrogenase-A from differently adapted vertebrates show no temperature-adaptive variation in G + C content. *Mol. Biol. Evol.* **20**, 105-110.

Rijkers, G. T., Frederix-Wolters, E. M. H. and Van Muiswinkel, W. B. (1980). The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). *Immunology* **41**, 91-97.

Small, J. V., Herzog, M. and Anderson, K. (1995). Actin filament organization in the fish keratocyte lamellipodium. *J. Cell Biol*. **129**, 1275-1286.

Somero, G. N. (1996). Biochemical mechanisms of cold adaptation and stenothermality in Antarctic fish. In *Biology of Antarctic Fish* (ed. G. di Prisco, B. Maresca and B. Tota), pp. 232-247. Berlin: Springer-Verlag.

Somero, G. N. and DeVries, A. L. (1967). Temperature tolerance of some Antarctic fishes. *Science* **156**, 257-258.

Somero, G. N., Giese, A. C. and Wohlschlag, D. E. (1968). Cold adaptation of the Antarctic fish *Trematomus bernacchii*. *Comp. Biochem. Physiol.* **26**, 223-233.

Theriot, J. A. and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. *Nature* **352**, 126-131.

Verlhac, V., Sage, M. and Deschaux, P. (1990). Cytotoxicity of carp (*Cyprinus carpio*) leucocytes induced against TNP-modified autologous spleen cells and influence of acclimatization temperature. *Dev. Comp. Immunol.* **14**, 475-480.